MOLECULAR PHARMACOLOGY, 22:20–25

MOL PHARM

Temperature Dependence of the Benzodiazepine-Receptor Interaction

ULRICH QUAST, HORST MÄHLMANN, AND KARL-OTTO VOLLMER

Department of Biochemistry, Gödecke Research Institute, Mooswaldallee 1-9, D-7800 Freiburg, Federal Republic of Germany

Received September 22, 1981; Accepted February 2, 1982

SUMMARY

The temperature dependence of the interaction of three benzodiazepines with their receptors in rat brain membranes containing about 2 μM (endogenous) γ-aminobutyric acid was investigated. van't Hoff plots of the equilibrium dissociation constants were linear for [^{3}H]diazepam and its *N*-desmethyl derivative (*N*-desmethyldiazepam, NDz), whereas for [^{3}H]flunitrazepam a break between two linear portions occurred at about 10°. Binding of diazepam, NDz, and flunitrazepam (for the latter, at temperature >10°) is enthalpy-driven ($\Delta H \approx -40$ to -50 kJ/mole with a negligible contribution from ΔS. The latter result indicates that the interaction is not a simple hydrophobic association, but that it may be more complex in nature, possibly involving a conformational transition of the receptor-ligand complex. The activation energy for dissociation of the [^{3}H]flunitrazepam- and [^{3}H]diazepam-receptor complexes is about 100 kJ/mole. Consequently, the complexes dissociate about 1000 times faster on changing temperature from 0° to 37°.

INTRODUCTION

Recent electrophysiological and biochemical investigations have shown that the benzodiazepines act (mainly) by facilitating GABA[1]-ergic transmission (for a recent review, see ref. 1). With the discovery of specific high-affinity binding sites for the benzodiazepines in the brain (2, 3), the first molecular step in the mode of action of these drugs has been elucidated. The classical benzodiazepines bind to a single class of noninteracting sites (e.g., see refs. 2 and 4). Recently, a second class of binding sites in brain has been described which has lower affinity for the centrally active benzodiazepines (5–8). These sites seem to be related to the peripheral (Ro 5-4864) sites (5–7). They do not contribute to the high-affinity binding discussed here. The further steps which transduce binding of the centrally active benzodiazepines into a potentiation of the GABA-ergic function remain unknown. We have recently shown by kinetic analysis that formation of the flunitrazepam-receptor complex involves a slow isomerization step which is preceded by a rapid binding step (8). This isomerization may reflect a conformational change involved in the chain of events which leads from binding to the physiological effect.

Thermodynamic analysis of the binding process may be a further means of gaining insight into the underlying molecular mechanism. In the first communications on the existence of a benzodiazepine receptor, attention was drawn to the strong temperature dependence of binding (9, 10). A detailed thermodynamic analysis of the [^{3}H]flunitrazepam-receptor interaction was made by Speth and colleagues (4). In this report we confirm the findings of these authors and extend the analysis on [^{3}H]diazepam and its *N*-desmethyl derivative (NDz). The latter compound was chosen because it is a major active metabolite after oral administration of several benzodiazepines (11). In the case of prazepam (12) and clorazepate (13), NDz is the main active species. We show here that binding of all three benzodiazepines is enthalpy-driven, and that changes in entropy upon binding are negligible.

MATERIALS AND METHODS

[^{3}H]flunitrazepam and [^{3}H]diazepam (specific activity of each, 88 Ci/mmole; radiochemical purity ≥98%) were purchased from New England Nuclear Corporation (Dreieich, West Germany). NDz was synthesized in our organic chemistry department.

Crude membrane fragments were prepared from whole rat brain by homogenization and two centrifugations at 49,000 × *g*. The membrane pellets were stored at −20° until use. A 50 mM phosphate buffer containing 0.9% NaCl and adjusted to pH 7.2 at the different temperatures was employed throughout. The membrane preparation contained about 0.2 nmole of endogenous GABA per milligram of tissue (wet weight) (8).

Equilibrium binding data. Equilibrium binding data for [^{3}H]flunitrazepam and [^{3}H]diazepam were obtained by incubating membrane fragments with various concentrations of the labeled ligand in triplicate to obtain saturation of the receptor sites from 5% to 95%. Incubation time varied from 150 min at temperatures below 10° to 20 min above 30°. Binding at 35° was stable for more than 30 min. The decrease in equilibrium binding with

Preliminary results of this study were presented at the 22nd Spring Meeting of the Deutsche Pharmakologische Gesellschaft, Mainz 1981 [Quast, U. *Naunyn-Schmiedebergs Arch. Pharmacol.* **316**:R68 (1981)].

[1] The abbreviations used are: GABA, γ-aminobutyric acid; NDz, *N*-desmethyldiazepam (*N*-descyclopropylmethylprazepam).

0026-895X/82/040020-06$02.00/0
Copyright © 1982 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

rising temperature was fully reversible upon subsequent cooling. The homogenates had a tissue concentration of 15 mg (wet weight) per milliliter in the case of [^{3}H] flunitrazepam and 25 mg/ml for [^{3}H]diazepam, corresponding to protein concentrations of 0.33 mg/ml and 0.55 mg/ml, respectively. Samples (1 ml) were rapidly filtered over Whatman GF/B filters, and the filters were washed with 15 ml of ice-cold buffer. Nonspecific binding was determined in the presence of 10μM unlabeled ligand and was typically 1.5–2% of total radioactivity. Filters were counted for ^{3}H after addition of 10 ml of Aqualuma Plus (J. T. Baker Chemical Company, Deventer, Holland) in a Packard Tri-Carb 3375 scintillation counter at 38% efficiency. Data were analyzed according to the method of Scatchard by (nonweighted) linear regression.

The binding of NDz was determined by competition with [^{3}H]flunitrazepam at a concentration of 1 nM. Displacement curves were evaluated by linear regression after linearization by log-logit transformation. The Hill coefficients were near-unity, as expected for a competitive interaction with one class of (non-interacting) binding sites. The EC_{50} values were corrected for the presence of [^{3}H]flunitrazepam according to the equation $K_I = EC_{50}/(1 + L^*/K^*)$, where K_I is the true equilibrium dissociation constant of NDz, EC_{50} is the concentration of NDz which displaces 50% of specific [^{3}H]flunitrazepam binding, L^* the concentration of free [^{3}H]flunitrazepam, and $K^* = K_D$ of flunitrazepam at the chosen temperature (e.g., see ref. 14). L^* ranged from 0.9 nM at low temperatures to 1.7 nM at higher temperatures, so that $1 + L^*/K^*$ ranged from 2.0 to 1.2 (see Table 1 for values of K^*).

Dissociation rates. At time zero, a 5000-fold excess of unlabeled ligand was added to 35 ml of thermostatically controlled solution containing receptor (~1 nM) and labeled ligand (1–2 nM). The decrease in concentration of the radioactive complex was followed by filtration. The kinetics was plotted as counts per minute (time) minus counts per minute (infinity) on a logarithmic scale versus time. The slope of the straight line yielded to the dissociation rate constant k_-.

Data analysis. Unweighted linear least-squares analysis and error estimation were performed according to statistical standard rules (15). Propagation of errors was calculated according to the method of Bevington (15).

RESULTS

Equilibrium binding of [^{3}H]flunitrazepam to benzodiazepine receptors was investigated at different temperatures from 0 to 40° in the presence (8) of about 2 μM endogenous GABA. Figure 1 shows some representative experiments plotted according to the method of Scatchard. The plots of the data correspond to straight lines. This indicates that the ligand binds to a single class of noninteracting sites and that the peripheral-type binding sites of lower affinity (5–8) do not come into play at the chosen ligand concentrations. With increasing temperature, the binding affinity decreased, as seen by the decreasing slopes of the Scatchard plots. These changes in affinity were fully reversible on lowering temperature, even after preincubation of the receptor-ligand complex at 30°, 35°, and 40° for 30 min and subsequent cooling to 0° (data not shown). The straight lines in Fig. 1 extrapolate toward the same abscissa intercept, indicating that the saturation value of specific binding, B_{max}, i.e., the total concentration of receptors, does not change with temperature. The parameters of all binding experiments are listed in Table 1. They are in excellent agreement with the data of Speth *et al.* (4) on the temperture dependence of [^{3}H]flunitrazepam binding.

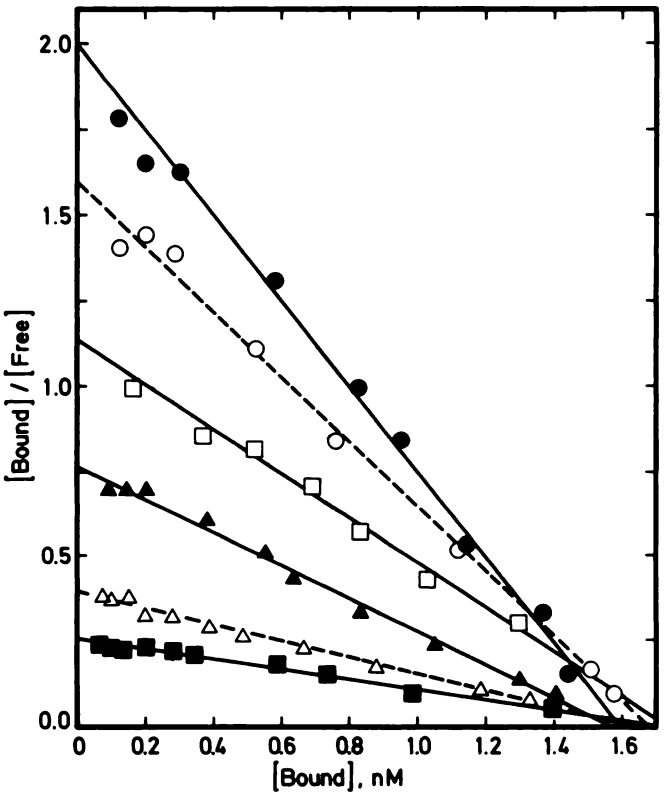

FIG. 1. *Binding of [^{3}H]flunitrazepam to benzodiazepine receptor in rat brain membranes at various temperatures*

The homogenate consisted of 15 mg of tissue (wet weight) per milliliter (≙ 0.33 mg of protein per milliliter) in 50 mM phosphate buffer containing 0.9% NaCl at pH 7.2. Data are presented according to the method of Scatchard. ●, 0°; ○, 10°; □, 15°; ▲, 20°; △, 30°; ■, 35°. For evaluation of parameters, see Table 1.

Similar results were obtained with [^{3}H]diazepam (see also Table 1). As with [^{3}H]flunitrazepam, rising temperatures led to a decrease in affinity but left maximal specific binding unchanged. As a third benzodiazepine, we examined NDz, since it is a key metabolite of several benzodiazepines used therapeutically (11). As this compound is not available in labeled form, its affinity toward the benzodiazepine receptor was determined by competition with 1 nM [^{3}H]flunitrazepam. The EC_{50} values were corrected for the presence of the label (see Materials and Methods), and the resulting K_I values are listed in Table 1. Maximal specific binding cannot be determined by this method. The data show that, again, the affinity decreased with increasing temperature.

In order to determine the changes in thermodynamic parameters occurring upon binding, the K_D values were plotted on a semilogarithmic scale versus T^{-1} (van't Hoff plot). As shown in Fig. 2, the data are fit by straight lines with the exception of those for flunitrazepam, where a small but significant deviation existed at temperatures

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

MOL PHARM

TABLE 1

Temperature dependence of equilibrium constants

Data for [^{3}H]flunitrazepam and [^{3}H]diazepam were obtained by Scatchard analysis of equilibrium binding experiments. The K_I values for NDz were determined by displacement of [^{3}H]flunitrazepam and are corrected for the presence of the competing radiolabel as detailed under Materials and Methods.

Temperature	[^{3}H]Flunitrazepam		[^{3}H]Diazepam		NDz
	K_D	B_{max}[a]	K_D	B_{max}[b]	K_I
	nM	*pmoles/mg protein*	*nM*	*pmoles/mg protein*	*nM*
0°	0.88 ± 0.04[c]	4.8 ± 0.6	3.1 ± 0.4	4.5 ± 0.4	9
5	0.98 ± 0.04	5.2 ± 0.3	4.2 ± 0.4	4.9 ± 0.4	
8					26
10	1.06 ± 0.06	5.2 ± 0.3	5.7 ± 0.7	4.9 ± 0.5	
15	1.60 ± 0.07	5.5 ± 0.3	6.4 ± 0.7	4.9 ± 0.5	35
20	2.1 ± 0.1	4.8 ± 0.6	8.0 ± 0.6	4.4 ± 0.5	60
25	3.2 ± 0.20	5.2 ± 0.9	12.2 ± 1.6	4.5 ± 0.9	76
30	4.4 ± 0.23	5.2 ± 0.9	17.8 ± 1.1	4.9 ± 0.7	123
35	6.7 ± 0.44	5.2 ± 0.9	22.3 ± 3.7	4.4 ± 0.9	80
40			30.4 ± 3.4	4.5 ± 1.3	

[a] B_{max} = concentration of binding sites in the homogenate [15 mg of tissue (wet weight) per milliliter corresponding to 0.33 mg of protein per milliliter]. On a molar basis, B_{max} was 1.7 nM.

[b] The homogenate consisted of 25 mg of tissue (wet weight) per milliliter (≙ 0.55 mg of protein per milliliter) and B_{max} was calculated to 2.6 nM.

[c] Mean value of three experiments. The standard deviations of the other values were determined from variance in linear regression of the Scatchard plot.

below 10°. The slopes equal $\Delta H/R$ (e.g., see ref. 16), so that the change in enthalpy, ΔH, is easily calculated. On the other hand, the free energy of binding, ΔG, is related to the equilibrium dissociation constant K_D by

$$\Delta G = RT \ln K_D$$

and the Gibbs equation connects ΔG and ΔH with the change in entropy ΔS:

$$\Delta G = \Delta H - T\Delta S$$

By combining these equations, ΔS is calculated and listed together with ΔH in Table 2. Comparison shows that, for all three benzodiazepines (for [^{3}H]flunitrazepam, consider values in the range >10°), there is a large negative change in enthalpy of about −45 kJ/mole and a small change in ΔS. This indicates that binding is enthalpy-driven (see Discussion).

The activation energies of association and dissociation, $E_a(+)$ and $E_a(-)$, can be determined from the temperature dependence of the respective kinetics. The dissociation kinetics of the receptor complexes with [^{3}H]flunitrazepam and [^{3}H]diazepam were measured after addition of a 5000-fold excess of unlabeled ligand to the respective (labeled) complex. Figure 3 shows some representative dissociation kinetics of the [^{3}H]flunitrazepam complex at various temperatures. The data are plotted in a normalized semilogarithmic form. At all temperatures, dissociation was monophasic, allowing determination of the dissociation rate constant k_-. In the case of flunitrazepam, measurements could be performed up to 35°, whereas with diazepam dissociation was considerably faster and measurements were feasible only up to 12°. An Arrhenius plot of the dissociation rate constants is shown in Fig. 4. The data are fit by straight lines, the slopes of which equal $-E_a(-)/R$. The corresponding activation energy values are 96 ± 4 kJ/mole (flunitrazepam) and 125 ± 17 kJ/mole (diazepam); see Table 2. These values are unusually high. Figure 2 shows that the rate of dissociation will increase about 1000-fold when going from 0 to 37°.

For a bimolecular reaction

$$A + B \underset{k_-}{\overset{k_+}{\rightleftharpoons}} C$$

the activation energies of association and dissociation are related to the change in enthalpy (e.g., see ref. 16) by

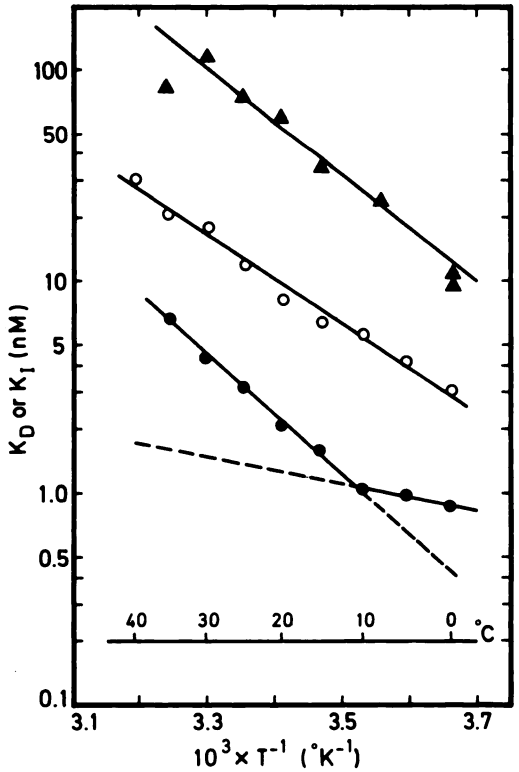

FIG. 2. *van't Hoff plot of equilibrium dissociation constants*

Data are from Table 1. ●, [^{3}H]Flunitrazepam: $T < 10°$: slope = −1500 ± 240 °K; $T > 10°$: slope = −6400 ± 310 °K. ○, [^{3}H]diazepam: slope = −4900 ± 230 °K. ▲, NDz: slope = −5250 ± 800 °K. The slopes in the van't Hoff plot equal $\Delta H/R$ with R = 8.3 J °K^{-1} mole^{-1} (gas constant).

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

TABLE 2

Thermodynamic parameters of benzodiazepine binding

Substance	ΔH^a	ΔS^b	$E_a(-)^c$	$E_a(+)^d$
	kJ/mole	*J/mole°K*	*kJ/mole*	*kJ/mole*
[^{3}H]Flunitrazepam (>10°)	−53 ± 3	−18 ± 23	96 ± 4	43 ± 5
[^{3}H]Flunitrazepam (<10°)	−13 ± 2	+124 ± 15		
[^{3}H]Diazepam	−41 ± 2	+17 ± 16	125 ± 17	84 ± 17
NDz	−44 ± 7	−10 ± 26		

[a] ΔH = change in enthalpy, determined from a van't Hoff plot (Fig. 2). The standard deviations were calculated from the variance of slope determined by linear regression (15).

[b] ΔS = change in entropy, calculated from $RT\ln K_D = \Delta H - T\Delta S$.

[c] $E_a(-)$ = activation energy for dissociation of receptor-ligand complex determined from an Arrhenius plot (Fig. 4).

[d] $E_a(+)$ = activation energy for association estimated from $E_a(+) = \Delta H + E_a(-)$.

$E_a(+) - E_a(-) = \Delta H$. With this equation, it was estimated that $E_a(+)$ is about 40 or 80 kJ/mole for flunitrazepam or diazepam, respectively (see Table 2).

DISCUSSION

In this communication we describe the temperature dependence of the interaction of three benzodiazepines with their binding sites in a crude membrane preparation from rat brain. In this preparation, some of the original GABA content of the brain was retained. At the final dilution, the GABA concentration of the membrane suspension was determined to 2.0 ± 0.5 μM (8). This is of importance since GABA is an allosteric modulator of benzodiazepine binding, increasing the affinity of the benzodiazepine receptor 2- to 3-fold (e.g., see refs. 17–19). At 0°, the residual amount of GABA present in this preparation is sufficient to shift 80 ± 10% of the benzodiazepine receptors to the high-affinity form (8), and a similar percentage is estimated at 37°. Therefore, the results discussed below refer to a receptor population with the larger part in the GABA-induced high-affinity form.

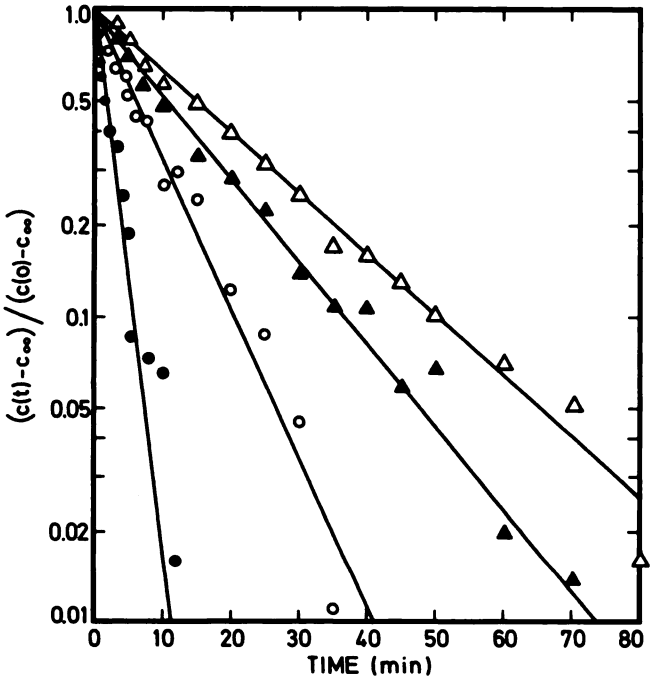

FIG. 3. *Dissociation kinetics of the [^{3}H]flunitrazepam-receptor complex at various temperatures*

Dissociation was initiated by addition of a 5000-fold excess of unlabeled ligand to labeled complex. Data are presented in a normalized semilogarithmic form [$c(t)$ = counts per minute at time t, c_∞ = counts per minute at equilibrium (= blank value)]. The slopes of the straight lines give the dissociation rate constants k_-: △, 0°, $k_- = 7.5 \times 10^{-4}$ sec^{-1}; ▲, 5°, $k_- = 1.1 \times 10^{-3}$ sec^{-1}; ○, 10°, $k_- = 1.7 \times 10^{-3}$ sec^{-1}; ●, 18°, $k_- = 7 \times 10^{-3}$ sec^{-1}.

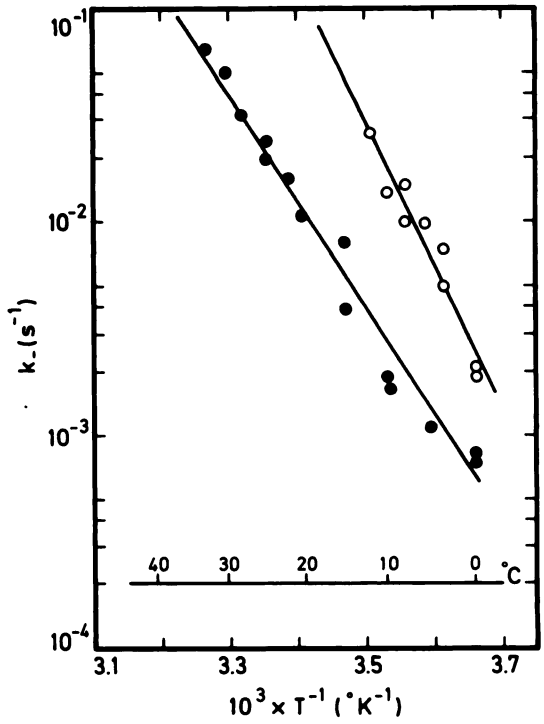

FIG. 4. *Arrhenius plot of the dissociation rate constants k_-*

●, Dissociation rate constants of the [^{3}H]flunitrazepam complex; ○, [^{3}H]diazepam complex. The slopes of the straight lines were determined by linear regression to $(-1.15 \pm 0.05) \times 10^4$ °K ([^{3}H]flunitrazepam) and $(-1.53 \pm 0.17) \times 10^4$ °K ([^{3}H]diazepam) and equal $-E_a(-)/R$.

Experimentally, this slight inhomogeneity of the receptor population is not expected to result in biphasic binding data. First, the difference in affinity between low- and high-affinity forms is rather small; second, simple mechanistic considerations, e.g., assuming a two-state scheme (e.g., see ref. 20), predict homogeneous binding data. Indeed, at all temperatures, binding of the three benzodiazepines examined revealed only one component (for flunitrazepam; see Fig. 1). The van't Hoff plots derived from these binding data were linear for diazepam and NDz, whereas a break was observed at 10° for [^{3}H]flunitrazepam (see Fig. 2). The data for [^{3}H]flunitrazepam agree with those of Speth *et al.* (4). Those authors discussed a phase transition of the membrane lipids or changes in micelle size and shape as possible reasons for the nonlinerity in the van't Hoff plot of [^{3}H]flunitrazepam binding. The observation of linear van't Hoff plots for [^{3}H]diazepam and NDz shows that the nonlinerity found with [^{3}H]flunitrazepam is not a general phenomenon in benzodiazepine receptor binding, but that it may indicate a special mode of binding of flunitrazepam. One

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

TABLE 3

Driving forces of benzodiazepine receptor binding at 37°

Substance	ΔG^a	ΔH^b	$T\Delta S^b$
	kJ/mole	*kJ/mole*	*kJ/mole*
[^{3}H]Flunitrazepam	−48 ± 6	−53 ± 3	−5 ± 7
[^{3}H]Diazepam	−45 ± 5	−41 ± 2	+5 ± 5
NDz	−41 ± 4	−44 ± 7	−3 ± 8

[a] Calculated from $\Delta G = RT\ln K_D$ with standard error estimated from the variance in linear regression.

[b] Values taken from Table 2.

might speculate that this behavior of flunitrazepam may be related to its ability in the receptor-bound state to form a covalent bond with the receptor after UV irradiation (21).

The main result of this study is that binding of the three benzodiazepines investigated is driven by a large loss in enthalpy. The enthalpic and entropic contributions to the free energy of binding at 37° are listed in Table 3. It can be seen that ΔG is dominated by ΔH, and that the entropic term $T\Delta S$, which can only be determined within considerable error, does not contribute significantly to ΔG.

The observed decrease in enthalpy means that energetically favorable contacts are formed in the complex. However, the negligible change in entropy is an unexpected result. As the three benzodiazepines studied here are very hydrophobic, the binding site of the receptor will also have similar properties. Consequently, these structures in their free state will be surrounded by a shell of ordered water molecules which is displaced when the ligand binds to the binding site. This release of structured water leads to a substantial increase in entropy which can be estimated to overcompensate the loss of translational and rotational entropy due to fixation of the ligand upon binding (e.g., see ref. 22). Therefore, the binding process is expected to result in a large increase in entropy, in contrast to the results listed in Table 3. However, the changes in thermodynamic parameters given there reflect over-all changes in the whole system consisting of solvent, ligand, and membrane-bound receptor coupled to other proteins (e.g., GABA-receptor and chloride ionophore). It is therefore assumed that the hydrophobic association step itself, which results in a large increase in entropy, is followed by other events which reverse this change in entropy and which are driven by a large decrease in enthalpy (for a quantitative discussion, see ref. 23).

In such a complex system as it is considered here, one can only speculate on the nature of the process which will reverse ΔS and ΔH. To cite a few examples, the decrease in entropy may be brought about, for example, by exposure of previously buried parts of the protein(s) to the solvent, by a loss of internal degrees of freedom of the protein(s), or by a (tighter) association of the components of the supramolecular (benzodiazepine receptor/GABA receptor/ionophore)-complex. At the same time, favorable contacts must be formed (e.g., between ligand and receptor, within the receptor itself, or between the different components of the supramolecular complex). This will lead to a decrease in enthalpy sufficiently large to compensate for the unfavorable loss in entropy. The over-all changes in thermodynamic parameters then show an enthalpy-driven process with a negligible change in entropy. One might speculate in more biological terms that the second step may be a conformational change in the receptor-ligand complex or a change in the coupling of the protein components of the supramolecular complex.

We have indeed obtained evidence that the interaction of [^{3}H]flunitrazepam with the benzodiazepine receptor is not a simple bimolecular process (8). A kinetic study at 0° revealed that a rapid binding step is followed by a slow isomerization of the complex (8). However, this is not a perfect case in point, since the kinetic experiments were conducted at 0°. At this temperature, flunitrazepam binding is largely entropy-driven [at 0°, $\Delta G = -47 \pm 3$ kJ/mole, $\Delta H = -13 \pm 2$ kJ/mole, and $T\Delta S = +34 \pm 4$ kJ/mole ($\Delta S = +124 \pm 15$ J/mole °K)]. Also, the kinetic mechanism of [^{3}H]flunitrazepam binding need not be the same on either side of the break point of the van't Hoff plot. Nevertheless, the thermodynamic data suggest that the interaction of benzodiazepines with their receptor may be a more complex process than simple adsorption of a ligand to a (rigid) receptor site.

There are some reports on the thermodynamic analysis of receptor-ligand interactions in the literature. In the case of membrane-bound nicotinic acetylcholine receptor from *Torpedo*, binding of the agonist choline was found to be entropy-driven [$\Delta H \approx 0$, $\Delta S = 10$ J/mole °K (24)]. With solubilized, purified acetylcholine receptor from *Electrophorus*, binding of agonists as well as antagonists was entropy-driven [$\Delta H \sim 80$ kJ/mole, $\Delta S \sim +400$ J/mole °K (25)]. Interesting studies were made by Weiland *et al.* on the interaction of agonists and antagonists with *beta*-adrenergic receptors in turkey erythrocyte membrane (26) and in various tissues of the rat (27). In both systems it was found that binding of antagonists was largely entropy-driven whereas binding of agonists was enthalpy-driven with a decrease in entropy. By argumentation similar to that which we have detailed above the authors inferred that binding of antagonists to the *beta*-receptor is a simple "hydrophobic" adsorption process. Binding of agonists may start with a similar step followed by an induced conformational change which reverses the over-all entropy balance and renders the interaction enthalpy-driven. The putative isomerization step might be involved in the agonist-induced activation of adenylate cyclase (26). In view of these thermodynamic differences in agonist and antagonist binding to the *beta*-adrenergic receptor(s) it seems of interest to extend the thermodynamic analysis presented here to the different classes of antagonists (28, 29) of the benzodiazepine receptor.

ACKNOWLEDGMENT

The help of Ms. S. Gärttner with some of the experiments is gratefully acknowledged.

Note added in proof. When this paper was being submitted, Möhler and Richards' communication (30) appeared in which the thermodynamics of binding of clonazepam and the antagonist Ro 15-1788 were described. The results for clonazepam (which, like flunitrazepam, possesses a nitro group in position 7) are qualitatively similar to those described here for flunitrazepam.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

Binding of Ro 15-1788 is mainly enthalpy-driven, with a small (15%) contribution of $T\Delta S$ to the energy of binding. The van't Hoff plott is linear. These observations partially support the speculations presented in this paper.

REFERENCES

1. Tallman, J. F., S. M. Paul, P. Skolnick, and D. W. Gallager. Receptors for the age of anxiety: pharmacology of the benzodiazepines. *Science* (*Wash. D. C.*) **207**:274–281 (1980).
2. Squires, R. F., and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature* (*Lond.*) **266**:732–734 (1977).
3. Möhler, H., and T. Okada. Benzodiazepine receptor: demonstration in the central nervous system. *Science* (*Wash. D. C.*) **198**:849–851 (1977).
4. Speth, R. C., G. J. Wastek, and H. I. Yamamura. Benzodiazepine receptor: temperature dependence of ^{3}H-flunitrazepam binding. *Life Sci.* **24**:351–358 (1979).
5. Schoemaker, H., M. Bliss, and H. I. Yamamura. Specific high-affinity saturable binding of [^{3}H]-Ro 5-4864 to benzodiazepine binding sites in the rat cerebral cortex. *Eur. J. Pharmacol.* **71**:173–175 (1981).
6. McCarthy, K. D., and T. K. Harden. Identification of two benzodiazepine binding sites on cells cultured from rat cerebral cortex. *J. Pharmacol. Exp. Ther.* **216**:183–191 (1981).
7. Gallager, D. W., P. Mallorga, W. Oertel, R. Henneberry, and J. Tallman. ^{3}H-Diazepam binding in mammalian central nervous system: a pharmacological characterization. *J. Neurosci.* **1**:218–225 (1981).
8. Quast, U., and H. Mählmann. Interaction of [^{3}H]flunitrazepam with the benzodiazepine receptor: evidence for a ligand-induced conformation change in benzodiazepine receptors. *Biochem. Pharmacol*, in press (1982).
9. Braestrup, C., and R. F. Squires. Specific benzodiazepine receptors in rat brain characterized by high-affinity ^{3}H-diazepam binding. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3805–3809 (1977).
10. Möhler, H., and T. Okada. Properties of ^{3}H-diazepam binding to benzodiazepine receptors in rat cerebral cortex. *Life Sci.* **20**:2101–2110 (1977).
11. Breimer, D. D., R. Jochemsen, and H. H. von Albert. Pharmacokinetics of benzodiazepines. *Arzneim. Forsch.* **30**:875–881 (1980).
12. Brodie, R. R., L. F. Chasseaud, and T. Taylor. Concentrations of *N*-descyclopropylmethylprazepam in whole blood, plasma and milk after administration of prazepam to humans. *Biopharm. Drug Dispos.* **2**:59–68 (1981).
13. Chun, A. H. C., P. J. Carrigan, D. J. Hoffman, R. P. Kershner, and J. D. Stuart. Effect of antacids on absorption of clorazepate. *Clin. Pharmacol. Ther.* **22**:329–335 (1977).
14. Weiland, G. A., and P. B. Molinoff. Quantitative analysis of drug-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29**:313–330 (1981).
15. Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences*. McGraw-Hill, New York, 55–65, 92–118 (1969).
16. Moore, W. J. *Physical Chemistry*, Ed. 5. Longman, London, Chaps. 3, 8, and 9 (1972).
17. Tallman, J. F., J. W. Thomas, and D. W. Gallager. GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* (*Lond.*) **274**:383–385 (1978).
18. Martin, I. L., and J. M. Candy. Facilitation of benzodiazepine binding by sodium chloride and GABA. *Neuropharmacology* **17**:993–998 (1978).
19. Karobath, M., and G. Sperk. Stimulation of benzodiazepine receptor binding by γ-aminobutyric acid. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1004–1006 (1979).
20. Janin, J. The study of allosteric proteins. *Prog. Biophys. Mol. Biol.* **27**:77–120 (1973).
21. Möhler, H., M. K. Battersby, and J. G. Richards. Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1666–1670 (1980).
22. Janin, J., and C. Chothia. Role of hydrophobicity in the binding of coenzymes. *Biochemistry* **17**:2943–2948 (1978).
23. Ross, P. D., and S. Subramanian. Thermodynamics of protein association reactions: forces contributing to stability. *Biochemistry* **20**:3096–3102 (1981).
24. Miller, J., V. Witzemann, U. Quast, and M. A. Raftery. Proton magnetic resonance studies of cholinergic ligand binding to the acetylcholine receptor in its membrane environment. *Proc. Natl. Acad. Sci. U. S. A.* **76**:3580–3584 (1979).
25. Maelicke, A., B. W. Fulpius, R. P. Klett, and E. Reich. Acetylcholine receptor: responses to drug binding. *J. Biol. Chem.* **252**:4811–4830 (1977).
26. Weiland, G. A., K. P. Minneman, and P. B. Molinoff. Fundamental difference between the molecular interactions of agonists and antagonists with the β-adrenergic receptor. *Nature* (*Lond.*) **281**:114–117 (1979).
27. Weiland, G. A., K. P. Minneman, and P. B. Molinoff. Thermodynamics of agonist and antagonist interactions with mammalian β-adrenergic receptors. *Mol. Pharmacol.* **18**:341–347 (1980).
28. Hunkeler, W., H. Möhler, L. Pieri, P. Polc, E. P. Bonetti, R. Cumin, R. Schaffner, and W. Haefely. Selective antagonists of benzodiazepine. *Nature* (*Lond.*) **290**:514–516 (1981).
29. Braestrup, C., and M. Nielsen. GABA reduces binding of ^{3}H-methyl β-carboline-3-carboxylate to brain benzodiazepine receptors. *Nature* (*Lond.*) **294**:472–474 (1981).
30. Möhler, H., and J. G. Richards. Agonist and antagonist benzodiazepine receptor interaction *in vitro*. *Nature (Lond.)* **294**:763–765 (1981).

Send reprint requests to: Dr. U. Quast, Department of Biochemistry, Gödecke Research Institute, Mooswaldallee 1-9, D-7800 Freiburg, Federal Republic of Germany.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012